

Preliminary investigation of a new serum marker for ovarian cancer

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Abstract

Objectives: The long-term survival rate of patients with carcinoma of the ovary is poor, because this condition is usually diagnosed at an advanced stage of the disease. A reliable diagnostic and screening test is still lacking. Therefore, a serological test for a novel ovarian tumour antigen was developed and investigated in a clinical setting. This report describes this investigation, the aim of which was to provide data to decide whether the test warrants a further, large-scale trial.

Methods: Serum collected from 25 patients with ovarian carcinoma, 24 healthy controls and 25 control patients with non-carcinomatous ovarian disorders was tested. The test utilises a monoclonal antibody, designated SMO47, to capture the tumour antigen and a normal form of the antigen from serum. Only the tumour antigen is detected by biotinylated *Maackia amurensis* lectin, which binds specifically to the sialic acid on the tumour antigen.

Results: The sensitivity for the carcinoma patient group was 60% and the specificity for the control patient group was 76% when a cut-off value for 100% specificity in the healthy controls was used. The area under the receiver-operator characteristic curve was 0.8200. CA125 tests were done on all serum, and the results compared graphically. The tumour antigen in the serum was very stable and did not seem to be affected by freezing or long storage at 4°C.

Conclusions: The results of this first application of the new test are encouraging and warrant further investigation and testing of larger numbers of subjects to obtain more significant values for the sensitivity and specificity.

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Introduction

Ovarian cancer is often referred to as the “silent killer”, because of late presentation and difficulty in diagnosis. Epithelial ovarian cancer is a common disease in countries with a larger elderly population. The incidence in American women aged 75-79 years is high at 57.3 /100 000 women/year.¹

Ovarian carcinoma can often be successfully treated if it is diagnosed at an early stage, but the diagnosis is often a death sentence because of late presentation and detection. The mortality associated with the diagnosis of epithelial ovarian cancer is invariably high, and statistics from the USA confirm that ovarian carcinoma is the most deadly gynaecological cancer after the age

of 45 years.¹ The inaccessible anatomical position of the ovaries makes it difficult to detect clinical signs. Clinical examination is therefore not very helpful in making the diagnosis, because many subtle signs may be missed.²

Background: screening with biomarkers

Ovarian cancer has a relatively low prevalence, and the risk of morbidity due to false positive tests may outweigh the benefits. For a good screening test with a minimum positive predictive value of about 10%, a specificity of greater than 99.6% and sensitivity greater than 75% would be necessary.

The most widely used biochemical marker for carcinoma of the ovary is the tumour antigen CA125 initially

described by Dr Robert Bast.³ At present, it is widely used as a marker of response to therapy.⁴ CA125 is secreted by coelomic and Müllerian epithelium, and a cut-off value of 30-35 units/ml is generally accepted as normal for postmenopausal patients.⁵ However, CA125 is a fairly non-specific screening test when used alone, and levels may be raised in various noncancerous conditions.⁶ CA125 may be raised in 50% of cases with stage I ovarian carcinoma, and in more than 90% of cases with advanced disease. There is generally a lead time of approximately 1.5-1.9 years from the time that the CA125 becomes elevated to the time of detection of clinical disease.

Multimodal screening strategies show the most promise. A UK group showed that sequential CA125 and transvaginal ultrasound may achieve specificity to detect ovarian carcinoma of 99.9%, with a positive predictive value of 26.8% (four operations/one case of ovarian carcinoma).^{7,8} Results from the long-awaited and very well-designed UKTOCS (United Kingdom Collaborative Trial of Ovarian Cancer Screening) study have recently been published.⁹ A large group of women were randomised to one of three groups in a ratio of 2:1:1. Half of the participants (n= 101 359) were randomised to no intervention, while the rest were divided between two screening strategies. One group (n=50 640) had annual CA125 measurements (interpreted with a risk of cancer algorithm), followed by transvaginal ultrasound if abnormal. This was referred to as the multimodal screening (MMS) group. Another group (n=50 639) underwent only annual transvaginal ultrasound. There was a significantly better specificity for detection of invasive epithelial ovarian and tubal carcinomas in the MMS group when compared to the ultrasound-only group. The morbidity cost, measured as the number of operations needed to diagnose one invasive cancer, was 35 operations per case in the ultrasound group and only three operations per case in the MMS group. The authors concluded that the sensitivity for both screening strategies were encouraging. However, before final conclusions can be reached about whether screening will change mortality, further study is needed. In reaction to these results, a group from Leuven in Belgium responded by calling the results "encouraging".¹⁰

The Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial that is ongoing in the USA aims to include 155 000 men and women, and the screening tool that will be utilised for ovarian cancer is CA125 combined with transvaginal scanning.¹¹ The results of this trial should be available in the next 5 to 10 years.

CA125 has also been used in combination other biochemical markers, for example OVX1 and macrophage colony-stimulating factor (M-CSF), to improve sensitivity.¹² OVX1 and M-CSF, in combination with CA125, increased the sensitivity from 66% to 76% to detect early-stage disease, but also decreased

the specificity from 96% to 83%. Of the large number of biomarkers that have been tested in the serum of patients with ovarian cancer, two promising tests were reported to be human epididymis protein 4 (HE4),¹³ and a soluble form of mesothelin.¹⁴ However, alone or in combination with CA125, they have also not been accepted as general screening tests. Other biomarkers mentioned in the literature include CA72-4,¹⁵ M-CSF,¹⁶ prostasin,¹⁷ osteopontin,¹⁸ inhibin,¹⁹ and human anterior gradient 2 (AGR2).²⁰ Despite all these potential candidates, there is still no single sensitive and specific biomarker for ovarian cancer.

Background: SMO47

The monoclonal antibody with clone designation SMO47, which is used in the assay described in this report, reacts well with formalin-fixed and routinely processed tissues, and its reactivity with tumour tissues²¹ and cytological preparations of peritoneal fluids²² has been described previously. It also reacts with normal epithelium of the respiratory tract, fallopian tubes, salivary glands, endometrium, uterine cervix, small bowel and ovary, and partly with mesothelium (RH Laeng, unpublished observations). The antigen to which SMO47 binds is a high-molecular-weight glycoprotein that occurs in a normal form in human male and female serum, and in a tumour-specific form in serum from female patients with carcinoma of the ovary and in spent culture medium of the ovarian carcinoma cell line UWOV1,²³ which expresses high levels of the antigen associated with the cellular glycocalyx. As was shown previously,²¹ the normal form is of heterogeneous molecular weight and contains either low level or no sialic acid residues, whereas the tumour-specific form is homogeneous, of higher molecular weight, and sialylated and sulphated.

Based on the previous findings of the high sialic acid content of the tumour antigen, the serological assay was designed to detect only the tumour antigen. The epitope to which the antibody binds seems to be repeated, as the antigen could be detected by an enzyme-linked immunosorbent assay (ELISA) in which the SMO47 antibody was used both as capture and detecting antibody (not shown). However, such an assay would detect both the normal and the tumour antigen. Therefore, *Maackia amurensis* lectin I (MAL1), which binds specifically to sialic acid in glycoproteins, was used to detect the sialic acid in the tumour antigen. Thus, although both antigens are captured by the plate-bound antibody, only the tumour antigen is detected.

Study design

Patients

Three separate groups of patients were included

in this study. Group A consisted of 25 patients with carcinoma of the ovary confirmed by histology. All patients in group A were newly diagnosed with carcinoma and patients with recurrent disease were not included. Group B consisted of 24 healthy female control subjects who were admitted for voluntary tubal ligation and underwent laparoscopy so that other subclinical peritoneal diseases could be excluded. (Patients requesting sterilisation, but with menstrual abnormalities or symptoms suggestive of pelvic infection, were excluded). Group C consisted of 25 patients with suspicions ovarian masses on preoperative assessment, but who had noncarcinomatous disorders on final diagnosis after surgical exploration. Patients were recruited preoperatively and, after informed consent was obtained, blood samples were collected for CA125 and SMO47 testing. The biochemist was blinded to all patients, and samples were identified by study numbers only.

The project was approved by the ethics committees of the University of Cape Town, where the monoclonal antibody was developed, and by the University of Stellenbosch, where the serological assay was developed and the patient sampling was performed.

Materials and reagents

The following materials and reagents were used: Maxisorp® ELISA plates from Nunc (Copenhagen, Denmark), Hybri-Max Protein-Free RPMI 1640® medium from Sigma (Kempton Park, South Africa), hydroxyapatite for chromatography from Bio-Rad (Marnes-la-Coquette, France), biotinylated MAL1 (2 mg/ml) and streptavidin peroxidase (1 mg/ml) from Vector Laboratories (Burlingame, CA, USA), and tetramethyl benzidine substrate from KPL (Gaithersburg, MD, USA). The murine immunoglobulin M (IgM) monoclonal antibody SMO47 for antigen capture was developed at the University of Cape Town from the spleen of a Balb/c mouse immunised with ovarian tumour tissue.

Purification of the SMO47 antibody

The hybridomas were cultured in protein-free RPMI 1640 medium, and the spent-culture medium passed through a hydroxyapatite column equilibrated with 10 mM sodium phosphate buffer pH 6.8.¹⁰ After washing with the buffer, the immunoglobulin was eluted with a 50-300 mM sodium phosphate gradient in 50 mM steps. The eluted antibody was tested electrophoretically for purity and was stored at 4°C with 0.05% sodium azide, or frozen in aliquots at -80°C.

Performance of the assay

The principle of the assay is shown diagrammatically in Figure 1. The blocking solution was 1% w/v skim milk

powder in phosphate-buffered saline (PBS), with 0.05% azide and the washing solution 0.1% v/v Tween 20 in PBS.

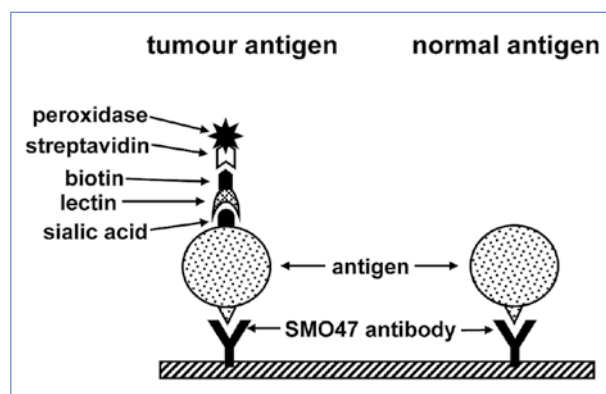


Figure 1: Principle of the SMO47 assay

ELISA plates were coated overnight at 4°C with SMO47 antibody at 10 µg/ml in PBS (50 µl/well) washed, and blocked for a minimum of one hour at room temperature. During the blocking step, the diluted serum and MAL1 were mixed in Eppendorf tubes, and incubated for one hour at room temperature (the final dilution of the serum in the mixture was 1/16, and that of the lectin, 1/320). Duplicate 50 µl aliquots were then dispensed into the blocked plates, and the plates placed on a slow rotary shaker for one hour at room temperature. After washing 50 µl/well, streptavidin-peroxidase diluted 1/500 in PBS without azide was added, the plate shaken for 30 minutes and, after washing again, 100 µl/well tetramethyl benzidine substrate. The reaction was stopped after six minutes with 100 µl/well of 1M phosphoric acid, and the absorbance at 450 nm recorded. The negative control was a pool of three normal sera, and the positive control was ascites from one of the patients with carcinoma of the ovary.

The serum of all the study participants was also tested for the CA125 tumour antigen with the Diasorin CA125 IRMA kit.

Expression of results

The results for the SMO47 assay were calculated by subtracting the mean background absorbance (serum substituted by PBS) from the means of duplicate test absorbances. These were divided by the mean of the ascites absorbance and multiplied by 1 000; that is, the ascites, which always gave the highest reading, was assigned 1 000 arbitrary units. As the test detected sialic acid residues on the tumour antigen and not tumour antigen molecules themselves, the use of a standard curve was not considered appropriate.

Results

Group A consisted of 25 confirmed cases of ovarian cancer, including serous (n=19), endometrioid (n=4), mucinous (n=1) and Sertoli-Leydig carcinoma (n=1), as shown in Table I.

Table I: Histological types of ovarian cancer encountered in the study

Histology	Number
Serous adenocarcinoma	19
Endometrioid adenocarcinoma	4
Mucinous adenocarcinoma	1
Sertoli-Leydig cell tumour	1
Total	25

The patients in group C all had a suspicious mass suggestive of ovarian cancer on preoperative evaluation, but exploratory surgery confirmed a wide range of pathologies (listed in Table II).

Table II: Final diagnoses of patients in Group C

Pathology	Number
Postinfective hydrosalpinges	7
Mucinous cystadenoma	2
Uterine fibroids	3
Benign cystic teratoma	2
Corpus haemorrhagicum	2
Endometrial carcinoma	2
Endometrioma	2
Benign Brenner tumour	1
Chronic ectopic pregnancy	1
Gastrointestinal tumour	1
Cystadenofibroma	1
Lymphoma	1
Total	25

Statistical analysis

The results for the patients with confirmed ovarian cancer (group A) were compared to those of the other groups by means of the Mann-Whitney test, and variation of sensitivity and specificity at different cut-off values was shown by means of a receiver-operator characteristic (ROC) curve from the data of the cancer patients and healthy controls. The GraphPad Prism 5 and Microsoft Office Excel® software was used for the graphical representations and statistical calculations.

As the stability and storage properties of the SMO47 tumour antigen were not known, serum was tested

blind soon after receipt, and then stored at 4°C with 0.05% azide and also frozen in aliquots at -20°C and -80°C. Tests were repeated several times on the unfrozen serum to assess the performance of the test and the stability of the antigen. The time between the collection of the first and last blood samples was two years and three months and the positive samples remained positive after storage at 4°C for this period, indicating good stability of the antigen. As it was a new assay, minor technical modifications were made during the course of the testing and, after receipt of ascites from patient number 48, this was used as reference for the expression of the results in preference to a normal serum pool used at the start of the testing. The initial tests could therefore not be analysed statistically. When blood collection was completed, aliquots of the serum stored frozen at -20°C were thawed and tested together in one ELISA test and, similarly, those frozen at -80°C. Those stored at 4°C were tested twice. All these tests were completed within nine days. As the antigen was stable during the two years of storage at 4°C and freezing also did not noticeably affect the results, the medians of the results of the four assays were used in the graphical representation of the results.

The ROC curve constructed from the data of the cancer patients and the healthy controls is shown in Figure 2. It shows that, when a cut-off value to give a specificity of 100% is chosen, the highest sensitivity of the test will be 60%. The area under the curve is 0.8200. The specificity for Group C at this cut-off value of 180 is 76%.

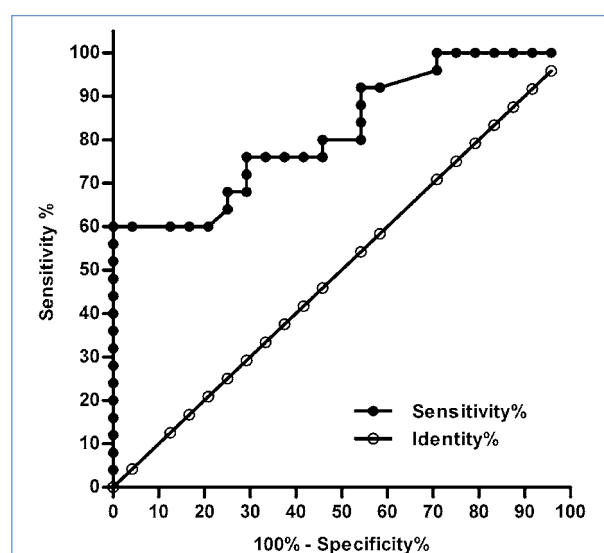


Figure 2: ROC curve constructed from the data of the cancer patients and healthy controls

The SMO47 test results for the three groups of subjects are shown in a scatter plot in Figure 3A, in which the dotted line is drawn at a cut-off value of 180 when the specificity

is 100% for the healthy controls and the sensitivity 60% for the carcinoma patient group. The results for cancer patients in Group A with the diagnoses are listed in Table III (false negatives in bold type), and the results for patients in Group C are listed in Table IV (false positives in bold type).

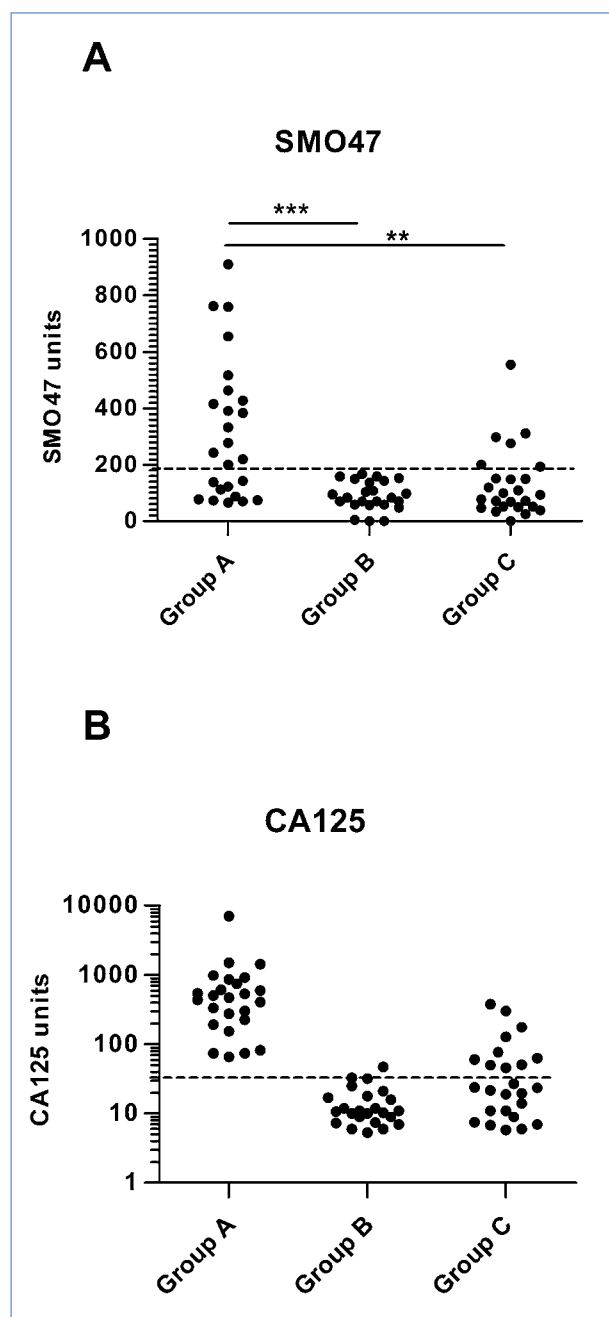


Figure 3: Test results for SMO47 (3A) and CA125 (3B) of the three groups of subjects

As the CA125 test is the most widely used serological assay in the diagnosis of carcinoma of the ovary, CA125 tests were done on the same serum and the results are shown in Figure 3B, where the dotted line is at the standard cut-off value or 35 units. The extent

Table III: Patients with ovarian carcinoma (Group A) (false negatives in bold)

Patient number	SMO47 result	Diagnosis
001	463	Serous adenocarcinoma ovary FIGO stage 3
002	220	Serous adenocarcinoma ovary FIGO stage 3
003	200	Endometrial carcinoma plus left carcinosarcoma FIGO stage 3
004	759	Endometrioid adenocarcinoma ovary with clear cells FIGO stage 3
005	113	Serous adenocarcinoma ovary FIGO stage 3
006	427	Serous adenocarcinoma ovary FIGO stage 3
010	88	Mucinous adenocarcinoma ovary FIGO stage 3
014	77	Serous adenocarcinoma ovary FIGO stage 3
018	391	Sertoli Leydig cell tumour FIGO stage 1
020	243	Endometrioid adenocarcinoma ovary FIGO stage 3
021	762	Serous adenocarcinoma ovary FIGO stage 1
022	143	Endometrioid adenocarcinoma ovary FIGO stage 2
026	278	Serous adenocarcinoma ovary FIGO stage 3
027	333	Serous adenocarcinoma ovary FIGO stage 3
029	415	Serous adenocarcinoma ovary FIGO stage 3
040	70	Serous adenocarcinoma ovary FIGO stage 3
048	910	Serous adenocarcinoma ovary FIGO stage 4
052	74	Serous adenocarcinoma ovary FIGO stage 3
055	516	Serous adenocarcinoma ovary FIGO stage 3
057	139	Serous adenocarcinoma ovary FIGO stage 2
064	73	Serous adenocarcinoma ovary FIGO stage 3
066	122	Endometrioid adenocarcinoma ovary FIGO stage 3
068	655	Serous adenocarcinoma ovary FIGO stage 4
070	66	Serous adenocarcinoma ovary FIGO stage 3
073	383	Serous adenocarcinoma ovary FIGO stage 3

Table IV: Patients with other pathologies (Group C) (false positives in bold)

Patient number	SMO47 result	Diagnosis
007	150	Papillary serous cystadenofibroma (benign)
008	69	Pelvic inflammatory disease
009	298	Pelvic inflammatory disease
019	50	Gastrointestinal tumour
023	554	Mucinous cystadenoma of ovary (benign)
024	0	Grade II follicular lymphoma
025	276	Benign cystic teratoma
028	38	Corpus haemorrhagicum and endometriosis
031	52	Uterine leiomyoma
034	110	Pelvic inflammatory disease
035	72	Endometrial adenocarcinoma (of the uterus)
036	51	Chronic inflammation
037	149	Borderline mucinous ovarian tumour
038	120	Papillary serous adenocarcinoma (of the endometrium)
041	25	Endometrioma (benign)
042	47	Cystic atypical Brenner tumour of ovary
043	34	Chronic ectopic pregnancy
076	152	Salpingitis
077	94	Uterine leiomyoma
078	77	Benign cystic teratoma
079	193	Haemorrhagic corpus luteum cyst
080	99	Uterine leiomyoma
083	311	Endometriosis
084	72	Endometrial polyp with chronic salpingitis
085	201	Acute-on-chronic salpingitis

of agreement between SMO47 and CA125 results is illustrated in Figure 4 for cancer patients (Figure 4A), healthy controls (Figure 4B) and control patients (Figure 4C). The dotted lines are at the cut-off value of 35 for CA125 and 180 for SMO47. The data points in the four quadrants made by the dotted lines thus show: the number of sera positive for both CA125 and SMO47 (upper right quadrant); those positive for CA125 and negative for SMO47 (lower right); those negative for CA125 and positive for SMO47 (upper left); and those negative for both tests (lower left). The diagnoses of patients in Group A whose sera were positive for CA125 and negative for SMO47 are listed in Table V.

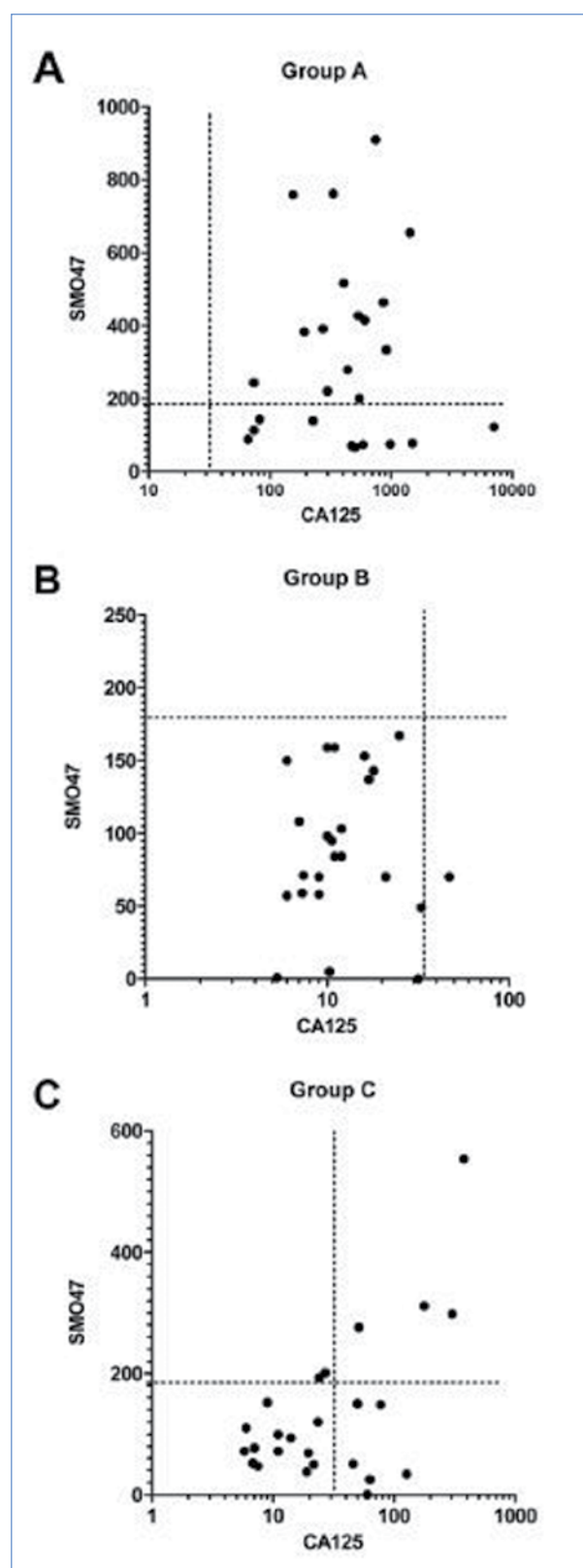
**Figure 4:** Extent of agreement between SMO47 and CA125 for patients with cancer (4A), healthy controls (4B) and control patients (4C)

Table V: Patients with ovarian carcinoma (Group A), positive for CA125 and negative for SMO47

Patient number	CA125	SMO47	Diagnosis
005	74.0	113	Serous adenocarcinoma ovary FIGO stage 3
010	65.9	88	Mucinous adenocarcinoma ovary FIGO stage 3
014	1 501.0	77	Serous adenocarcinoma ovary FIGO stage 3
022	81.9	143	Endometrioid adenocarcinoma ovary FIGO stage 2
040	471.7	70	Serous adenocarcinoma ovary FIGO stage 3
052	986.0	74	Serous adenocarcinoma ovary FIGO stage 3
057	228.0	139	Serous adenocarcinoma ovary FIGO stage 2
064	588.0	73	Serous adenocarcinoma ovary FIGO stage 3
066	7 061.0	122	Endometrioid adenocarcinoma ovary FIGO stage 3
070	505.0	66	Serous adenocarcinoma ovary FIGO stage 3

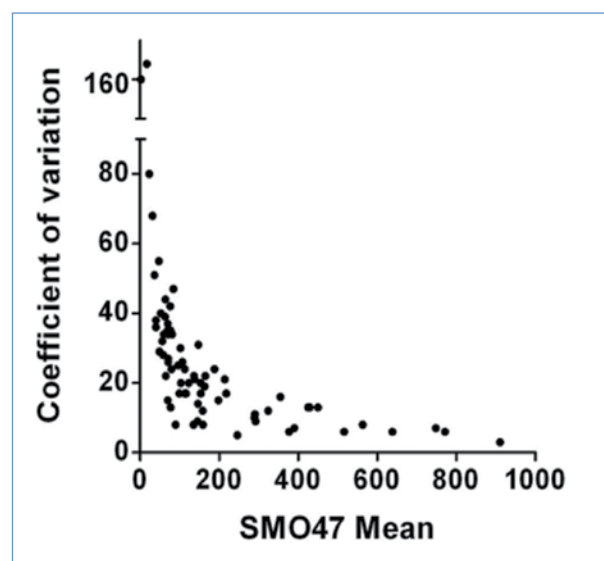
Discussion

At present, there is no effective, single biomarker for screening for ovarian cancer, and the results of the exploratory study presented here are therefore of interest. The SMO47 assay did not detect some of the ovarian carcinomas and was positive in some patients with other pathological conditions, but this is also seen with tests for other markers. The assay had a good specificity in normal subjects.

The effect of sampling in small numbers of patients is illustrated by the finding of 100% sensitivity for CA125 in our group of patients with carcinoma. It is well established in general clinical practice that sensitivity is only approximately 80% overall.^{4,12}

The function of the normal SMO47 antigen is not known, and the purpose of sialylation by the tumour cells could be to avoid recognition by immune cells, because sialic acids can act as biological masks.²⁴ On the other hand, sialylated and sometimes sulphated recognition structures could trigger responses by cells of the immune system and other leukocytes, platelets and endothelium that express proteins known to bind sialic acid. These proteins are the selectins, which have been associated with the spread of epithelial

tumour cells via the bloodstream.²⁵ Siglecs (sialic acid-recognising immunoglobulin superfamily lectins) are a subfamily of sialic acid-binding proteins that share homology with variable and constant domains of immunoglobulins (e.g. CD22, CD33 and sialo-adhesin) which are expressed differentially in cells of the haematopoietic system.²⁶ Siglecs have two conserved motifs resembling immune-receptor tyrosine-based inhibition motifs (ITIMS) in their cytoplasmic tails, and may thus be involved in down-regulation of the activated immune system.

**Figure 5:** The coefficients of variation of the four repeated tests for each serum sample versus means of the test results

The reasons why the sialylated antigen is not found in the bloodstream of 40% of the patients with cancer in this study would be of interest in relation to the prognosis and the metastatic potential of the tumour. Of relevance in this respect could be an interesting finding that humans, who can produce the sialic acid *N*-acetylneuraminic acid (Neu5Ac) but not *N*-glycolylneuraminic acid (Neu5Gc) (as a result of a deletion mutation in the *CMAH* gene), can metabolically incorporate the latter from red meat and dairy products in the diet.²⁷ This can then trigger immune responses to the non-human Neu5Gc, which is the mechanism of the "serum sickness" reaction described by Hanganutziu and Deicher early in the 20th century. These Hanganutziu-Deicher (HD) antibodies were found to react with Neu5Gc bound to horse serum glycoconjugates, and were then discovered in the serum and tissues of patients with cancer²⁸ and in activated and malignant immune cells.²⁹ Antibodies of varying isotypes were found in many or all normal human subjects,^{27,29} and their binding to cells can result in complement-mediated cytotoxicity. If some ovarian cancer patients have HD antibodies, they could be responsible for neutralising the secreted SMO47

antigen, if it contains Neu5Gc. (MAL1 binds to both Neu5Ac and Neu5Gc.)³⁰

The limitations of this study are the following:

- The number of subjects tested in each group of patients is too small to make significant statistical comparisons, and an assessment of its clinical value cannot be made until after a further larger-scale study.
- The cut-off value of 180, which was chosen to ensure 100% specificity in the healthy control subject group, may not be optimal.
- The methodology of the test is completely new and may not be optimal yet.

However, we suggest some positive aspects of the study:

- The test achieved a reasonable negative predictive value in the group of patients with other pathologies.
- The SMO47 antibody reacts well with routinely processed tissue sections,²¹ and immunocytochemistry can complement the test findings.
- The tumour antigen is stable. The coefficients of variation of the assay repeats at four times are plotted versus the means of the tests in Figure 5.

Conclusion

In conclusion, the chemical nature of the normal and tumour-associated SMO47 antigen is largely unknown. The serological assay for SMO47 must be studied in larger numbers of cancer patients and normal subjects in order to assess its performance and clinical relevance.

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